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Deoxyribonucleic Acid Fingerprinting Methods for *Candida* Species

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Summary

A number of genetic fingerprinting methods have evolved to analyze the population structure and to perform epidemiological and etiological studies of infectious fungi. These methods include multilocus enzyme electrophoresis, restriction fragment-length polymorphism using complex probes, random amplification of polymorphic DNA, and multilocus sequence typing, which are described in this chapter.

Key Words: DNA fingerprinting; restriction fragment-length polymorphism (RFLP); complex probes; random amplification of polymorphic DNA (RAPD); multilocus sequence typing (MLST).

1. Introduction

Genetic fingerprinting has become an indispensable tool in understanding the population structure, epidemiology, and etiology of fungal diseases ***(1,2)***. Several effective methods have evolved for genetic fingerprinting, including multilocus enzyme electrophoresis (MLEE), restriction fragment-length polymorphism (RFLP) with or without a probe, random amplification of polymorphic deoxyribonucleic acid (RAPD), multilocus sequence typing (MLST), and a variety of other methods. All but the first also are referred to as DNA-fingerprinting methods. Several important points must be kept in mind concerning genetic fingerprinting ***(1)***. First, the method must provide the resolution necessary to answer the question(s) posed. Second, the method must be verified for such resolution by comparison with an unrelated genetic fingerprinting method. Third, the method must be economical, specifically for large epidemiological questions, and within the capabilities of the experimenter. Finally, the data should be comparable with similar data collected by others. Here, we describe

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three DNA fingerprinting methods that are both popular and generally effective: RFLP with probe, RAPD, and MLST.

2. Materials

2.1. Preparation of Genomic DNA

1. Yeast peptone dextrose (YPD) medium: 1% yeast extract, 2% peptone, 2% dextrose.
2. Sorbitol potassium phosphate (SPP): 1 *M* sorbitol, 50 m*M* potassium phosphate, pH 7.4. Make 50 m*M* of potassium phosphate by mixing 40.1 mL of 1 *M* K_2HPO_4 with 9.9 mL of 1 *M* KH_2PO_4 in 1 L of total volume.
3. Zymolyase solution: 100 mg of Zymolyase 20T (Seikagaku America, Ijamsville, MD) in 800 µL of 50 m*M* sodium phosphate, pH 6.5, and 50% glycerol.
4. Bursting buffer: 50 m*M* Tris-HCl pH 7.4, 20 m*M* ethylene diamine tetraacetic acid (EDTA).
5. 10% Sodium dodecyl sulfate (SDS).
6. 5 *M* Potassium acetate.
7. 1:1 Phenol/chloroform.
8. Isopropanol.
9. Absolute ethanol.
10. TE: 10 m*M* Tris-HCl, pH 7.5; 1 m*M* EDTA.
11. 10 mg/mL RNase A (Sigma, St. Louis, MO).

2.2. Probing a Genomic DNA Southern Blot

1. DNA molecular weight markers (Promega, Madison, WI).
2. 1X TBE: 10X TBE is 108 g of Tris base, 55 g of boric acid, and 20 mL of 0.5 *M* EDTA, pH 8.0, in 1 L of total volume.
3. Random Primer Labeling Kit, (Life Technologies, Gaithersburg, MD).
4. Sephadex G-50 column (5'-3', Boulder, CO).
5. DNA loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll type 400 in sterile water.
6. *Eco*RI restriction enzyme.
7. Molecular biology-grade agarose.
8. Hybond N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ).
9. Calf thymus DNA: prepare a 10 mg/mL solution by dissolving calf thymus DNA (sodium salt; Sigma) in sterile water. Shear the DNA by sonicating at 50 W for 15-s bursts, 5–10 bursts (Sonics and Materials, Inc., Danbury, CT). Check the fragment size by running a 1-µL aliquot on an agarose gel. The average size fragment should be <2 kb in length.
10. 20X SSPE: 3.6 *M* NaCl, 0.2*M* NaH_2PO_4, and 20 m*M* EDTA. Adjust pH to 7.0.
11. Hybridization buffer: 5X SSPE, 5% dextran sulfate, 0.3% SDS. Store at 20°C.
12. Wash solution: 2X SSPE, 0.2% SDS.
13. Autoradiography film.

2.3. Fingerprinting Genomic DNA by MLST

1. Taq DNA Polymerase and appropriate buffer (Invitrogen, Carlsbad, CA).
2. 2 m*M* (each) Deoxynucleotide 5'-triphosphate (dNTPs) (deoxyadenosine 5'-triphosphate, deoxythymidine 5'-triphosphate, deoxycytidine 5'-triphosphate [dCTP], and deoxyguanosine 5'-triphosphate).
3. 2 n*M* Oligonucleotide primers.
4. 50 m*M* Magnesium chloride.
5. Sterile distilled water.
6. 5X sequencing buffer: 80 m*M* Tris-HCl, pH 9.0, 2 m*M* magnesium chloride.
7. Commercial kit for polymerase chain reaction (PCR) product purification (e.g., Genelute PCR Clean Up Kit, Sigma).
8. BigDye Terminator Cycle Sequencing Ready Reaction Mix Version 1 (Applied Biosystems, Foster City, CA).
9. Absolute ethanol.
10. 3 *M* Sodium acetate.
11. GlycoBlue (3.75 g/L) coprecipitant (Ambion, Austin, TX).

2.4. Fingerprinting Genomic DNA by RAPD

1. Taq DNA Polymerase and appropriate buffer (Invitrogen).
2. 0.2 m*M* (each) dNTPs (deoxyadenosine 5'-triphosphate, deoxythymidine 5'-triphosphate, dCTP, and deoxyguanosine 5'-triphosphate).
3. RAPD 10-mer primer kits (Operon Technologies, Alameda, CA).
4. 1X TBE: 10X TBE is 108 g of Tris-HCl base, 55 g of boric acid, and 20 mL of 0.5 *M* EDTA pH 8.0, in 1 L of total volume.
5. Molecular biology-grade agarose.
6. DNA loading dye: 0.25% bromophenol blue, 0.25% xylene cyanol, 15% Ficoll type 400 in sterile water.
7. 40 µg/L of ethidium bromide solution.

3. Methods

3.1. RFLP With a Probe

RFLP with a complex DNA fingerprinting probe has been demonstrated to be a very effective method for analyzing large samples, providing information relevant to population structure, as well as microevolution ***(1,3)***. This protocol is performed in two steps. The first involves separating genomic DNA on an agarose gel and then fixing it to a nylon membrane. The second involves hybridization with a DNA probe. The result is a complex pattern that is amenable to computer analysis. The DNA probe must either be available for the species of interest or a DNA probe must be isolated, characterized and confirmed as an accurate measure of genetic distance (*see* **Note 1** ***[4–9]***).

3.1.1. Preparation of Genomic DNA

1. Grow a 2-mL culture of *Candida* spp. in YPD broth to stationary phase (10^8 cells/mL). Harvest the cells by centrifugation at 15,000*g* in a Microfuge for 3 min and wash the pellet one time with 1 mL of sterile water (*see* **Note 2**).
2. Wash the pellet once with 0.7 mL of SPP. Resuspend the pellet in 0.6 mL of SPP containing 0.1% β-mercaptoethanol. Add 15 µL of Zymolyase solution and incubate the cell suspension for 30–90 min at 37°C.
3. Assess spheroplasting by microscopic examination and by lysing 10 µL of cells on a microscope slide with 1 µL of 10% potassium hydroxide. Spheroplasts lyse, but cells with intact walls do not. When >80% of the cells have become spheroplasts, pellet them at 15,000*g* for 5 min at room temperature (*see* **Note 3**).
4. Wash the spheroplast pellet once with 0.7 mL SPP. Resuspend the pellet in 0.6 mL of bursting buffer by repeatedly drawing the pellet into a 1 mL pipet until the solution is homogeneous. Do not vortex! Add 60 µL of 10% SDS and incubate for 30 min at 65°C. Add 240 µL of 5 *M* potassium acetate, shake the tube gently by hand, and incubate for 1 h on ice.
5. Centrifuge the cell lysates at 15,000*g* for 10 min at 4°C. Recover the supernatant and extract once with an equal volume of phenol/chloroform. Do not vortex. Precipitate the nucleic acids with an equal volume of isopropanol. Wash twice with 0.7 mL of 75% ethanol. Do not centrifuge the DNA pellet for more than 30 s.
6. Dry the nucleic acid pellet on the bench top and resuspend it in 100 µL of TE.
7. Remove the contaminating RNA by adding 2 µL of RNase A and incubating for 1 h at 37°C. If desired, extract once with an equal volume of phenol/chloroform and precipitate as in **step 5**. Resuspend the pellet in 100 µL of TE.

3.1.2. Probing a Genomic DNA Southern Blot

1. Digest 3 µg of the *Candida* genomic DNA with *Eco*RI or another appropriate restriction enzyme in a 30-µL final volume following the manufacturers instructions. Use a threefold excess of enzyme and allow the reactions to take place for at least 4 h. Add 3 µL of DNA loading dye to each reaction.
2. Separate the genomic DNA on a 0.8% agarose 1X TBE gel run at 120V until the bromophenol blue dye has migrated 18 cm from the origin. Run the same strain in the outside lanes of each gel so that the gels can be later calibrated to each other (*see* **Note 4**).
3. Transfer the gel to a Hybond N+ membrane by Southern blotting. After Southern blotting, fix the DNA to the Hybond N+ membrane by exposing the membrane, DNA side up, to ultraviolet light for 3.5 min (*see* **Note 5**).
4. Place each blot into a separate-heat sealable bag with 5 mL of prewarmed hybridization buffer containing 0.1 mg/mL heat-denatured calf thymus DNA. It is desirable to seal the bag so that it is only slightly larger than the blot; this will concentrate the hybridization buffer as well as the probe. Prehybridize for 6–16 h at 65°C in a shaking water bath.

5. Prepare ^{32}P-dCTP labeled DNA probe following the instructions provided by the manufacturer of the random primer labeling kit. Pass the probe over a Sephadex G-50 column to remove any unincorporated labeled nucleotides and count the finished probe in a scintillation counter. Add approx 5×10^6 cpm of labeled probe to the blot without changing the hybridization buffer. Let the blot hybridize overnight.
6. Pour off the hybridization buffer and probe and wash the blot 3×20 min in 500 mL of wash solution at 45°C in a sealed plastic container in a shaking water bath. If multiple blots were hybridized, they can be washed together.
7. Seal each blot in plastic wrap and expose to autoradiographic film for 1–3 d depending on the strength of the radioactive signal.
8. As long as the blot has not dried completely, it can be stripped of radioactive label and used again. These blots can be stored in plastic wrap at room temperature.

3.1.3 Analyzing the Repetitive DNA Probe Data

For Southern blot hybridization, patterns generated by moderately repetitive sequences, two similarity coefficients (S_{AB}s) are useful, one based on the position and intensity of bands and one based on position alone.

1. S_{AB} based on position and intensity:

$$S_{AB} = \frac{\left(a_i + b_i - \left|a_i - b_i\right|\right)}{\sum_{i=1}^{k}\left(a_i - b_i\right)}$$

   where a_i and b_i are the intensities of bands in patterns A and B, respectively, and k is the number of bands. If the patterns of strains A and B are identical (all bands are matched in size [in kilobases according to their migration] and of the same intensity), the S_{AB} will be 1.00. If no bands match, the S_{AB} will be 0.00. Increasing matches with increasingly comparable intensities results in S_{AB} ranging from 0.01 to 0.99.
2. S_{aB} based on position alone:

$$S_{AB} = 2E/(2E + a + b)$$

   where E is the number of bands in patterns A and B, which are the same size, a is the number of bands in pattern A with no correlate in pattern B, and b is the number of bands in pattern B with no correlates in pattern A. Again, this value will vary according to the complexity of the patterns that are compared. In the case of *Candida albicans* fingerprinted with the moderately repetitive probe Ca3, the average S_{AB} for unrelated isolates is 0.69 ± 0.11 ***(3)***. This average will vary according to the complexity of the patterns that are compared.

The S_{AB} can be computed between every pair of isolates and a matrix of similarity coefficients generated. Arbitrary thresholds can be assigned for unrelated, moderately related, and highly related patterns, but the best way to assess relatedness for a collection of independent isolates is through the genesis of a dendrogram based on S_{AB}s. Dendrograms are most easily generated based on the unweighted pair group method ***(10)***.

3.2. Multilocus Sequence Typing

3.2.1. Choice of Loci To Be Used

MLST is a new, although effective, methodology. Where information is already available on the variability of genes or their protein products for a certain species, it may be possible to select loci to use for MLST directly. However, if there is no information on variability, it is necessary to screen a number of genes in a panel of unrelated isolates to determine which are suitable for use with MLST. It is important to choose genes that are not under selective pressure, as they will bias any results obtained for population genetic analysis. For this reason genes known to code for virulence factors or resistance determinants should not be included. The number of loci to be used in the final scheme will depend on the variability seen and the level of discrimination required, but as a guide, six to eight should be sufficient (*see* **Note 6** ***[11,12]***).

3.2.2. PCR Primer Design

Primers can be designed using a variety of commercial packages, such as PrimerSelect (Dnastar, Inc., Madison, WI). Primers for a particular gene should be separated by between 500 and 650 bp to allow for a 450–600-bp sequence to be obtained. Although primers may be designed that fall within noncoding regions, they must amplify enough coding DNA for analysis, as only coding DNA sequence is deemed useful for analysis. Desirable features of primer pairs include, similar melting temperatures (T_m) for both primers in the pair and relatively high T_m (as primers with low T_m will require low annealing temperatures to be used, resulting in a greater likelihood of nonspecific annealing and spurious product formation). If the variability of the sequence of the locus in question is known, primers should be designed to be within conserved regions of the gene and to encompass variable regions (*see* **Note 7**).

3.2.3. Amplification of Desired Loci

1. In a microfuge tube, mix 2.5 µL of 10X PCR buffer, 1 µL of dNTPs (2 m*M* each), 2.5 µL of each PCR primer (2 n*M*), 1 µL of $MgCl_2$ (50 m*M*), 0.2 µL of *Taq* DNA polymerase (5 U/µL), and 15.8 µL of sterile distilled water with 1 ng of template DNA (in 1 µL).

2. Amplify the target DNA in a thermal cycler using the following reaction conditions: 7 min at 94°C, 30 cycles of: 1 min at 94°C, 1 min at an annealing temperature appropriate to the primers used (typically 50–55°C) and 1 min at 74°C. Finish with one 10-min cycle at 74°C.
3. Purify the PCR product following the manufacturers instructions (Genelute PCR Clean Up Kit, Sigma).

3.2.4. Sequencing

All loci should be sequenced in both the forward and reverse directions. The same primers as those used for the PCRs may be used.

1. Perform the sequencing reactions in a 20-µL volume with 1.5-µL oligonucleotide primer (2 n*M*), 25-ng template, 4 µL of BigDye Terminator Cycle Sequencing Ready Reaction Mix, and 2 µL of 5X sequencing buffer.
2. The thermal cycling conditions are as follows: 4 min at 96°C followed by 25 cycles of 5 s at 96°C and 4 min 5 s at 60°C.
3. Remove excess dye by ethanol precipitation. Place the 20 µL of sequencing reaction product in a fresh 1.5-mL tube and add 80 µL of water, 300 µL of 100% ethanol, 5 µL of 3*M* sodium acetate, and 2 µL of GlycoBlue (3.75 g/L) coprecipitant. Incubate for 30 min at room temperature before pelleting by centrifugation for 20 min at 15,000*g* in a microfuge. Wash the pellet with 500 mL of 70% ethanol for 15 min, followed by centrifugation at 15,000*g* for 20 min. Remove the supernatant and dry the pellet at 100°C.

The reaction products may be analyzed using an automated DNA sequencer according to the manufacturer's instructions (*see* **Note 8**).

3.3. Random Amplification of Polymorphic DNA

3.3.1. Principle

The RAPD approach is a frequently used method and is particularly effective for fingerprinting organisms for which DNA sequence data is limited or unavailable ***(13,14)***. In this method, genomic DNA is amplified by PCR using single, short oligonucleotide primers (10 bp). The polymorphisms analyzed consist of differences in the size of the DNA fragments amplified between samples. The choice of the primers used is usually empirical. Ideally, for a given micro-organisms, a panel of 20–40 primers is tested on a limited collection (3 or 4 isolates) and the best primers, the ones giving polymorphic bands that can be analyzed with no ambiguity and are reproducible, are selected to analyze larger collections (*see* **Notes 9–11**).

3.3.2. RAPD-PCR Amplification and Banding Pattern Visualization

All the primers used should be analyzed after identical PCR protocols. It is usually easier to test different primers rather than to try to optimize the conditions for specific primers.

1. Prepare the template DNA following the instructions provided in **Subheading 3.1.1.**
2. PCR is performed in 0.5-mL microcentrifuge tubes containing 25 µL of the following reaction mixture: 1 ng of genomic DNA; 2.5 µL of 10X buffer provided for the *Taq* DNA polymerase used; 1 U of *Taq* polymerase; 0.2 m*M* (each) dNTPs; 0.4 µ*M* of one of the primers used, and sterile water to make up the volume (*see* **Notes 12–14**).
3. DNA amplifications are performed in a thermal cycler programmed for 45 cycles of 1 min at 94°C, 1 min at 36°C, and 2 min at 73°C, followed by a final 10-min step at 73°C.
4. Add 3 µL of DNA loading dye solution to each PCR.
5. Separate amplification products on a 1.5% agarose 1X TBE gel run at 110 V for 4 h so that the bromophenol blue marker dye migrates approx 10 cm.
6. Stain the gel with ethidium bromide by placing the gel in a solution of ethidium bromide for 1 h with agitation.
7. The bands are visualized using an ultraviolet light box and the pattern is recorded photographically (*see* **Notes 15** and **16**).
8. The equation given in **Subheading 3.1.3.**, **step 2** can be used for analysis of DNA bands generated by RAPD.

4. Notes

1. Good complex probes are available for a number of *Candida* species. Check the literature for availability of the probe of choice. Probes can also be manufactured for any species as long as good high-molecular weight DNA can be made from the organism ***(9)***. Protocols can be found in Girardin et al. ***(4)***, Joly et al. ***(5,7)***, Lockhart et al. ***(6,9)***, and Enger et al. ***(8)***.
2. This general methodology can be used for any species of fungi as long as good high-molecular weight DNA can be made from the organism.
3. If the yeast cells are not forming spheroplasts very efficiently, then β-glucuronidase or Novozyme (both from Sigma) can be tried instead of or in addition to Zymolyase 20T.
4. Based on the average size fragment to which the probe hybridizes, the percentage of agarose in the Southern blot gels can be adjusted to separate or compact the hybridization bands.
5. Hybond N+ membrane is preferred for Southern blotting because it is easy to strip of radioactivity and reprobe with a second probe. Because it is nylon, it is durable.
6. The methodology described is suitable for fingerprinting species with haploid genomes. Though it may be used for diploid species, some additional steps may

enhance its utility. It can be anticipated that some of the loci studied in diploid species will be heterozygous. By sequencing direct from PCR products, these heterozygosities will be shown by double peaks in the sequencing traces. Thus, where heterozygosities exist at multiple nucleotide sites in any particular locus, assignment of individual alleles is not possible. This may be overcome by cloning the PCR product into a suitable vector and sequencing again from the vector. As only one of the alleles will be present in the vector, its sequence may then be determined and the sequence of the other allele may then be inferred by deduction.

7. MLST was originally designed for use with bacteria. The high numbers of polymorphisms found within bacterial species meant that only coding DNA sequences were used in these schemes, as the use of the even more polymorphic noncoding sequences may have led to homoplasy (the presence of identical characters in distinct phylogenetic lineages that are not acquired by descent, but rather through convergence, parallelism or reversion) complicating the results obtained. However, for fungi where the differences within a species are much less marked, this may not be the case, and noncoding sequences may have a role to play
8. A number of software packages exist for the analysis of the data obtained by MLST. Some of these may be downloaded for free at www.mLst.net. These include BURST, which uses the allele data to assess the relationships between sequence types; a program for the calculation of the index of association, and thus assessment of the likelihood of recombination within the population; and NRDB, which compares sequence data in order to assign alleles. Alternatively, the START package (available for free at http://outbreak.ceid.ox.ac.uk/software.htm) includes BURST, and index of association calculation, as well as a number of other features, such as the construction of dendrograms, tests for selective pressure, and the ability to export data in formats useable by other packages. The free package PHYLIP (http://evolution.genetics.washington.edu/phylip.htmL) is useful for phylogenetic analysis and the construction of dendrograms. The commercial package PAUP may be used for population genetic analysis (http://paup.csit.fsu.edu/). The commercially available Bionumerics (Applied Maths, Austin, TX) package is useful for the construction of databases containing sequence data, and the construction of dendrograms..
9. The power of this method increases with the number of primers used and the number of polymorphic bands analyzed per micro-organism. We recommend that eight primers or a total of 15–20 polymorphic bands be used to obtain data useful for cluster analysis. This may vary for any given species.
10. 10-mer custom oligonucleotides may be purchased from different companies. However, special attention should be paid to the purity of the oligonucleotides. High-performance liquid chromatography or polyacrylamide gel electrophoresis purification will be necessary for reproducible results. We found that the panels of RAPD primer kits commercialized by Operon Technologies represent a good alternative. Their panels of 1200 available primers have been selected for sequences containing a G+C content of 60–70% with no self-complementary ends. The majority of these primers work well with the PCR conditions proposed here, and for a number of different species.

11. A lack of reproducibility among laboratories, but also within the same laboratory, has somewhat hindered the use of the RAPD method. The banding patterns have been shown to be strongly affected by even slight differences in the PCR procedure ***(15–18)***. These problems can be circumvented, at least for intralaboratory reproducibility, by controlling carefully PCR procedures and the following recommendations.
12. Always use the same *Taq* DNA polymerase.
13. The stability of the patterns may depend on the concentration of the template DNA used. To test for this possibility, different concentrations of template DNA should be tested. Primers that generate patterns that are very sensitive to the template concentration should be discarded.
14. Control DNAs that have already been characterized for a given primer should always be run in parallel with new samples.
15. Independent DNA extractions of the same isolates should show the same patterns. If this is not the case, the primers showing differences should be discarded.
16. Avoid analyzing all of the bands obtained. Some of the bands obtained with a primer may be reproducible, whereas others will not be. In general, most of the bands that do not show reproducibility are low-intensity bands. One must be cognizant of this possible pitfall in analyzing such bands.

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References

1. Soll, D. R. (2000) The ins and outs of DNA fingerprinting the infectious fungi. *Clin. Microbiol. Rev.* **13,** 332–370.
2. Soll, D. R., Lockhart, S. R., and Pujol, C. (2003) Laboratory procedures for the epidemiological analysis of microorganisms, in *Manual of Clinical Microbiology 8th Ed.* (Murray, P. R., Baron, E. J., Pfaller, M. A., Tenover, F. C., and Yolken, R. H., eds.), ASM Press, Washington D. C., pp. 139–161.
3. Pujol, C., Joly, S., Lockhart, S. R., Nöel, S., Tibayrenc, M., and Soll, D. R. (1997) Parity among the randomly amplified polymorphic DNA method, multilocus enzyme electrophoresis, and Southern blot hybridization with the moderately repetitive DNA probe Ca3 for fingerprinting *Candida albicans*. *J. Clin. Microbiol.* **35,** 2348–2358.
4. Girardin, H., Latgé, J., Srikantha, T., Morrow, B., and Soll, D. R. (1993) Development of DNA probes for fingerprinting *Aspergillus fumigatus*. *J. Clin. Microbiol.* **31,** 1547–1554.
5. Joly, S., Pujol, C., Schröppel, K., and Soll, D. R. (1996) Development of two species-specific fingerprinting probes for broad computer-assisted epidemiological studies of *Candida tropicalis*. *J. Clin. Microbiol.* **34,** 3063–3071.

6. Lockhart, S. R., Joly, S., Pujol, C., Sobel, J., Pfaller, M. A., and Soll, D. R. (1997) Development and verification of fingerprinting probes for *Candida glabrata*. *Microbiology* **243,** 3733–3746.
7. Joly, S., Pujol, S., Rysz, M., Vargas, K., and Soll, D. R. (1999) Development and characterization of complex DNA fingerprinting probes for the infectious yeast *Candida dubliniensis*. *J. Clin. Microbiol.* **37,** 1035–1044.
8. Enger, L., Joly, S., Pujol, C., Simonson, P., Pfaller, M., and Soll, D. R. (2001) Cloning and characterization of a complex DNA fingerprinting probe for *Candida parapsilosis*. *J. Clin. Microbiol.* **39,** 658–669.
9. Lockhart, S. R., Pujol, C., Joly, S., and Soll, D. R. (2001) Development and use of complex probes for DNA fingerprinting the infectious fungi. *Med. Mycol.* **39,** 1–8.
10. Sneath, P. and Sokal, R. (1973) *The Principles and Practice of Numerical Classification*. W. H. Freeman & Co., San Francisco.
11. Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., et al. (1999) Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc. Natl. Acad. Sci. USA*. **95,** 3140–3145.
12. Bougnoux, M. E., Morand S., and d'Enfert, C. (2002) Usefulness of multilocus sequence typing for characterization of clinical isolates of *Candida albicans*. *J. Clin. Microbiol.* **40,** 1290–1297.
13. Welsch, J., and McClelland, M. (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucleic Acids Res.* **18,** 7213–7224.
14. Williams, J. G., Kubelik, A. R., Livak, K. J., and Tingey, S. V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* **18,** 6531–6535.
15. Ellsworth, D. L., Rittenhouse, K. D., and Honeycutt, R. L. (1993) Artifactual variation in randomly amplified polymorphic DNA banding patterns. *Biotechniques* **14,** 214–217.
16. Loudon, K. W., Coke, A. P., and Burnie, J. P. (1995) "Pseudoclusters" and typing by random amplification of polymorphic DNA of *Aspergillus fumigatus*. *J. Clin.Pathol.* **48,** 183–184.
17. Loudon, K. W., Coke, A. P., Burnie, J. P., Lucas, G. S., and Liu Yin, J. A. (1994) Invasive aspergillosis: clusters and sources? *J. Med. Vet. Mycol.* **32,** 217–224.
18. Meunier, J. R., and Grimont, P. A. (1993) Factors affecting reproducibility of random amplified polymorphic DNA fingerprinting. *Res. Microbiol.* **144,** 373–379.

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